



IJCRR  
Section: Healthcare  
Sci. Journal Impact Factor: 6.1 (2018)  
ICV: 90.90 (2018)



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# Invitro Study to Analyse and Evaluate the Antimicrobial Activity of Coelomic Fluid of Earthworm *Octochaetona suriensis* (Michaelsen, 1910)

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## ABSTRACT

**Introduction:** Earthworms are true coelomates, metamerically segmented annelids. The coelomic fluid of Earthworms is said to have demonstrated antimicrobial activity.

**Objectives:** To study the antimicrobial activity of different concentrations (5 µl, 10 µl, 15 µl, 20 µl/ disc) of coelomic fluid of Earthworm *O. suriensis* (*Octochaetona suriensis*) against standard ATCC (American type culture cells /colonies) strains of microbes like *S. aureus* (*Staphylococcus aureus*), *E. faecalis* (*Enterococcus faecalis*), *E. coli* (*Escherichia Coli*), *P. aeruginosa* (*Pseudomonas aeruginosa*) and *C. Albicans* (*Candida albicans*).

**Methods:** A standard antibiotic disc of AMP (Ampicillin) 10 µg/disc was used as a positive control for bacteria and KT (Ketoconazole) 10 µg/disc for fungi. A plane 6mm Whatman's filter paper disc was used as a negative control on a Petri plate, that was incubated overnight at 37°C for bacteria and 25°C for fungi and results were tabulated.

**Results:** The coelomic fluid of *O. suriensis* has exhibited excellent antimicrobial activity against all ATCC strains of microbes even at minimum concentrations. In *P. aeruginosa* it has shown 20mm zone of inhibition at 5µl and at 37 mm zone of inhibition 20 µl, while standard antibiotic Ampicillin did not exhibit any zone of inhibition. In *C. albicans* it has shown 11mm zone of inhibition at 20µl which is very near to inhibition shown by positive control (ketoconazole 10µg/disc) 12mm.

**Conclusion:** We conclude that coelomic fluid of earthworm *O. suriensis* is a potent antimicrobial substance. The molecular study should be carried out that may help us to discover new antimicrobial agents.

**Key Words:** Earthworms, Antimicrobial activity, Coelomic fluid, *Octochaetona suriensis*, Antimicrobial substances, Antimicrobial resistance

## INTRODUCTION

Earthworms are ancient creatures which inhabited this planet at least 700 million years ago.<sup>1</sup> Earthworm are annelids belonging to class "Oligochaeta". These are commonly called as bait worms, rain dew worms, farmer's friend and nature's ploughman.<sup>2</sup> Earthworms are tube-shaped hermaphrodites which have the characteristic feature of metamerous segments<sup>3</sup>, which means they are segmented externally as well as internally. These worms are true coelomates and their body cavity is called as coelom which is filled with coelomic fluid. The coelomic fluid of earthworms is said to have demonstrated antimicrobial ac-

tivity according to the study conducted by many researchers in recent past.

The mesoderm of earthworms has a cavity called a coelomic cavity which is filled with coelomic fluid.<sup>4</sup> Coelomic fluid of earthworms helps them in burrowing, respiration, immune response against pathogens etc. The coelomic fluid consists of coelomocytes which play important role in their innate immunity.<sup>5</sup> The innate immunity of earthworms depends on coelomocytes that synthesize and secrete antimicrobial molecules.<sup>6</sup> There are four types of coelomocytes namely, amoebocytes, mucocytes, circular cells and chlorogogen cells which vary in size shape and functions. Apart from

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**ISSN: 2231-2196 (Print)**

**ISSN: 0975-5241 (Online)**

**Received: 18.07.2020**

**Revised: 23.09.2020**

**Accepted: 12.11.2020**

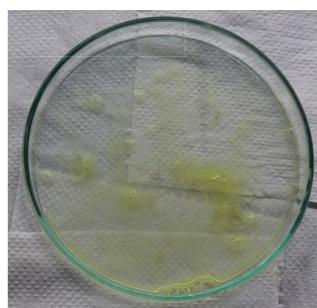
**Published: 14.12.2020**

coelomocytes, the coelomic fluid comprises of watery matrix and plasma. The coelomic fluid of earthworms contains leukocytes and biologically active molecules that participate in phagocytosis and encapsulation. It is presumed that they secrete effectors modulators of the innate immune response such as cytotoxic proteins and antibacterial molecules.<sup>7</sup> In the coelomic fluid, the antimicrobial activity is ascribed to some proteins such as lysozymes and fetidines.<sup>8-10</sup>

In recent times microorganisms such as bacteria and fungi affecting human and plants have developed high antibiotic resistance which is resulting in various resistant strains of bacteria and fungi. Hence Antibiotic resistance is emerging as a medical menace. Due to the lack of development of newer antibiotics to combat such resistant strains, current medical research calls for screening and evaluation of compounds that show promising antimicrobial activity and focus on the discovery of novel antibiotic compounds which could help to address such emerging challenges about antibiotic resistance.

The public and plant health is compromised, due to this mortality rates are increasing causing a global emergency to look into research for the discovery of novel antibiotic compounds that can address such challenges, this has become a virtual reality due to the huge finances and inherent challenges revolving around drug discovery. Since the early sixties, Coelomic fluid of earthworms has demonstrated several biological activities such as antimicrobial, cytolytic etc<sup>9</sup>. Several species of earthworms such as *Eudrilus eugeniae*, *Eisenia fetida*, *Lampito mauritii*, *Dichogaster bolaui* etc have been studied to demonstrate antimicrobial activity in various regions of India and other countries.

For the present study, the earthworm selected is *Octochaetona suriensis* which is a native species belonging to Kalaburagi district Karnataka, India. This worm is brownish-black in colour with a body length of 65-80 mm as in (Figure 2). The coelomic fluid of *O. suriensis* is a straw-coloured or fluorescent yellow sticky fluid as in (Figure 1), which dries up when kept open for a longer time. Each earthworm oozes out approximately 25 ml of coelomic fluid from its dorsal pores.



**Figure 1:** Physical appearance of fresh Coelomic fluid extract from *Octochaetona suriensis*.



**Figure 2:** Picture of *Octochaetona suriensis* in a washing tray.

## MATERIALS AND METHODS

### Collection of Earthworms

The earthworms were collected using digging and hand sorting method<sup>11</sup> for present study, worms were kept in a separate collection and storage basket with proper aeration and their habitat soil, required temperature, moisture was maintained conducive for their viability. This method for collection of earthworms is widely used for earthworm sampling<sup>12</sup> the earthworm in the present study is *octochaetona suriensis*, these worms were collected from a cotton field located in Chittapur taluka of Kalaburagi district, Karnataka, India.

### Collection of Coelomic Fluid

#### Electric shock method

The collected earthworms were fed with tissue papers for 48 hours to eliminate gastrointestinal metabolites and contaminants. They were then washed under running tap water in a tray and rapidly dried on a filter paper. These worms were then subsequently excited with 5 volt electric stimulations to produce coelomic fluid through their dorsal pores<sup>13</sup>. The collected coelomic fluid was then used for antimicrobial activity.

### Collection of pathogenic bacteria for antimicrobial studies

ATCC strains of commonly occurring microbes were selected with their known pathogenesis and drug resistance profile, these were obtained from Department of microbiology, Raja Rajeswari Medical College and Hospital, Bangalore. The pathogenic bacteria such as *Staphylococcus aureus*, *Enterococcus Faecalis*, *Escherichia Coli*, *Pseudomonas aeruginosa* and pathogenic fungi *Candida albicans* are tested against the collected coelomic fluid of *Octochaetona suriensis*. The details of the microbes used for the analysis of antimicrobial activity are given in table 1.

**Table 1: Microbial strains against the coelomic fluid of *Octochaetona suriensis***

SL. No	List of Microbe Tested (ATCC STRAINS)
1	<i>Staphylococcus aureus</i> (ATCC 25923)
2	<i>Enterococcus Faecalis</i> (ATCC 2912)
3	<i>Escherichia Coli</i> (ATCC 25922)
4	<i>Pseudomonas aeruginosa</i> (ATCC 27853)
5	<i>Candida Albicans</i> (ATCC 1023)

The in-vitro antimicrobial activity was screened against *Staphylococcus aureus* (ATCC 25923), *Enterococcus Faecalis* (ATCC 2912), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) by using Muller Hinton Agar (MHA). To prepare the MHA plates, sterile Petri plate was taken and 15ml of molten media was poured into it, these plates were allowed a resting time of 5 minutes for solidification. Using a sterile swab, 0.1 % of the inoculum was swabbed uniformly over the Petri plate and allowed to dry for five minutes. Subsequently, sterile autoclaved 6mm Whatman's filter paper disc was placed on the surface of the medium. Four concentration of the coelomic fluid (5 µl, 10 µl, 15 µl, 20 µl / disc) of *Octochaetona suriensis*, were loaded on 6mm sterile Whatman's filter paper disc. The different concentrations of coelomic fluid loaded in the disc were allowed to diffuse for 5 minutes. The plates were then kept for incubation at 37 degrees Centigrade for 24 hours. At the end of incubation, Inhibition zones formed around the disc were measured with standard zone reader scale in mm (millimetre). The bacterial inoculum was matched with 0.5 McFarland 10<sup>8</sup> CFU/ml adding sterile nutrient broth, before incorporating bacteria ( $\lambda=625$  nm). A disc of Ampicillin (AMP) (10 µg/disc) was used as a positive control and a plane 6mm Whatman's filter paper disc was used as a negative control.

### Antifungal assay

The in-vitro antifungal activity was screened against *Candida albicans* (ATCC 10231), This microorganism was inoculated on Sabouraud Dextrose broth during 24 hours at 25°C. The inoculate absorbance was established between 1 McFarland 10<sup>8</sup> CFU/ml by adding Sabouraud Dextrose broth before the fungal incorporation was carried out ( $\lambda=530$  nm). Subsequently, this fungal strain was seeded on the Sabouraud Dextrose agar by using 4% dextrose later the 6mm sterile Whatman's filter paper disc was seeded onto the agar plate. These discs were loaded with four different concentrations of the coelomic fluid (5 µl, 10 µl, 15 µl, 20 µl / disc). Ketoconazole (KT) (10 µg/disc) was used as a positive control and a sterile 6mm Whatman's filter paper disc was used as a negative control. The Petri plate was incubated at 25°C for 48 hours. The diameter of the zone of inhibition was measured with a standard zone reader scale and recorded accordingly.

## RESULTS

The coelomic fluid from *O. suriensis* was tested against four standard ATCC (American Type Culture Collection) strains of bacteria and one standard ATCC strain of fungi. Viz., *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa* and one ATCC strain of fungi named *Candida albicans*. The observed zone of inhibition exhibited by the coelomic fluid of earthworm *O. suriensis* against the positive and negative control is tabulated and the observed values are furnished as in (Table 2).

**Table 2: Antimicrobial activities of *Octochaetona suriensis* against standard ATCC strains of bacteria and fungi in millimetres (mm) at different concentrations**

Microbes Tested	Concentrations of Coelomic Fluid of <i>O. Suriensis</i> Tested in Millimeters (Mm)				Positive Control Ampicil- lin (10Mg/ Disc)	Negative Control
	5µL	10µL	15µL	20µL		
<i>S. aureus</i>	7	9	10	11	36	-
<i>E. faecalis</i>	7	9	10	12	24	-
<i>E. Coli</i>	7	8	11	13	18	-
<i>P. aerugi- nosa</i>	20	31	35	37	-	-
<i>C. albicans</i>	8	9	10	11	12	-

The activity of different concentrations of coelomic fluid of *O. suriensis* was evaluated against selected ATCC strains of four Bacteria and one Fungi. Four different concentrations (5 µl, 10 µl, 15 µl and 20 µl) were used. Against standard ATCC strain of *S. aureus*, the coelomic fluid of *O. suriensis* has exhibited maximum inhibition at a concentration of 20 µl (11 mm) as in (Figure 3) and minimum zone of inhibition at 5 µl (7 mm) concentration (Figure 3). Similarly, against *E. faecalis* it has shown maximum activity at 20 µl (12 mm) and minimum zone of inhibition at 5 µl (7 mm) as in (Figure 4). For *E. coli* (13 mm) was the zone of inhibition at 20 µl concentrations and minimum zone of inhibition at 5 µl (7 mm) concentrations (Figure 5). The coelomic fluid of *O. suriensis* has shown maximum zone of inhibition in *P. aeruginosa* at all the concentrations with a maximum zone of inhibition at 20 µl (37mm) followed by a minimum zone of inhibition at 5 µl (20 mm) and no zone of inhibition with the positive control (Figure 6). As evident by the table above, against *C. Albicans* the maximum zone of inhibition was shown at concentration 20 µl (11 mm) which is very near to inhibition shown by positive control (ketoconazole 10 µg/disc) (12 mm) and minimum inhibition was seen at concentration of 5 µl (8 mm) (Figure 7).



**Figure 3:** Antibacterial activity of Coelomic fluid of *O. suriensis* against standard ATCC strains of *S. aureus* with positive control of Ampicillin (AMP) (10 $\mu$ g/disc) and Negative control as plain 6mm Whatman's filter paper disc



**Figure 6:** Antibacterial activity of Coelomic fluid of *O. suriensis* against standard ATCC strains of *P. aeruginosa* with a positive control of Ampicillin (AMP) (10 $\mu$ g/disc) and Negative control as plain 6mm Whatman's filter paper disc



**Figure 4:** Antibacterial activity of Coelomic fluid of *O. suriensis* against standard ATCC strains of *E. faecalis* with positive control of Ampicillin (AMP) (10 $\mu$ g/disc) and Negative control as plain 6mm Whatman's filter paper disc



**Figure 7:** Antibacterial activity of Coelomic fluid of *O. suriensis* against standard ATCC strains of fungi *C. albicans* with positive control of Ketoconazole (KT) (10 $\mu$ g/disc) and Negative control as plain 6mm Whatman's filter paper disc.



**Figure 5:** Antibacterial activity of Coelomic fluid of *O. suriensis* against standard ATCC strains of *E. coli* with a positive control of Ampicillin (AMP) (10 $\mu$ g/disc) and Negative control as plain 6mm Whatman's filter paper disc.

## DISCUSSION

Numerous studies done in the recent past have demonstrated antimicrobial activity. Although coelomic fluid of different species of earthworms has been studied, the specific substance that is responsible for antimicrobial activity in a coelomic fluid needs to be studied further.

In this study we attempted to evaluate the antimicrobial activity of earthworm coelomic fluid prepared from *O. suriensis* for its antimicrobial activity, the results obtained from the experiment shows that, when the coelomic fluid was observed against lawn culture of *S. aureus* with standard comparator antibiotic Ampicillin 10  $\mu$ g/disc, it exhibited maximum zone inhibition at a concentration of 20  $\mu$ l (11mm), 15  $\mu$ l (10mm), 10  $\mu$ l (9mm) and minimum zone of inhibition at 5  $\mu$ l (7mm) concentration against standard antibiotic Ampicillin 10  $\mu$ g/disc (36mm) zone of inhibition. Hence against *S. aureus* with an increase in the concentration of coelomic fluid above 20  $\mu$ l may give better results.<sup>11</sup>

Similarly, against *E. faecalis* with standard comparator antibiotic Ampicillin 10 µg/disc, it exhibited maximum zone inhibition at a concentration of 20 µl (12mm), 15 µl (10 mm), 10 µl (9 mm) and minimum zone of inhibition at 5 µl (7mm) concentration against standard antibiotic Ampicillin 10 µg/disc (24 mm) zone of inhibition. In *E. faecalis* the zone of inhibition at 20 µl (12mm) is half of the standard antibiotic used. Hence it's evident that although the coelomic fluid of *O. suriensis* demonstrated good activity at 20 µl (12mm) against *E. faecalis*. By increasing the concentrations of coelomic fluid and technique modifications may enhance the potency of the coelomic fluid in further studies.<sup>12-15</sup>

Against *E. coli*, the coelomic fluid of *O. suriensis* has exhibited zone of inhibition at concentrations 20µl (13mm), 15 µl (11mm), 10 µl (8mm) and minimum zone of inhibition at 5µl(7mm) concentration against standard antibiotic Ampicillin 10 µg/disc (18 mm)zone of inhibition. This shows that the coelomic fluid of *O. suriensis* has demonstrated excellent activity at concentrations 20 µl (13mm), against standard antibiotic Ampicillin 10 µg/disc (18 mm). Therefore, further research in the same with advanced techniques may give promising results towards combating *E. coli* resistance.<sup>14,15</sup>

The Petri plate containing lawn culture of *P. aeruginosa* inoculated with the coelomic fluid of *O. suriensis* as compared with standard antibiotics shows following zones of inhibition at concentrations 20 µl (37mm), 15 µl (35mm), 10 µl (31mm)and minimum zone of inhibition at 5µl (20mm) concentration respectively. The standard antibiotic Ampicillin 10 µg/disc showed no zone of inhibition. Hence it is evident that coelomic fluid of *O. suriensis* has great potency against *P. aeruginosa* even at least concentrations of 5µl (20mm). Therefore, further studies may lead to the discovery of a novel compound responsible for antimicrobial activity in the coelomic fluid of *O. suriensis*.

In Petri plate containing lawn culture of *C. albicans*, the coelomic fluid of *Octochetona suriensis* as compared with standard antibiotics shows following zones of inhibition at concentrations 20 µl (11mm), 15 µl (10mm), 10µl (9mm) and minimum zone of inhibition at 5µl(8mm) concentration respectively, whereas the standard antibiotic Ketoconazole, exhibited 12mm zone of inhibition. From the above values, we can say that coelomic fluid of *O. Suriensis* demonstrated excellent fungicidal activity at concentrations 20 µl (11mm) which is very near to standard antibiotic.

## CONCLUSION

The coelomic fluid of *O. suriensis* has shown definite activity against standard ATCC (American Type Culture Collection) strains of bacteria *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa* and fungi *C. Albicans* even at the least concentra-

tion of 5µl/disc and some visible activity at a concentration equivalent to 20µl/disc compared with strain-specific antibiotic discs used as the positive control. This gives us a lead to carry out further research with clinically relevant ATCC (American Type Culture Collection) strains of more bacteria and fungi, wherein coelomic fluid of *O. suriensis* could be used in higher concentrations and antimicrobial activity noted. This study also lays down the foundation for carrying out detailed research to understand the molecular mechanism that interplay in inhibiting or killing the microorganisms, it also mandates detail study to find out the active compound, its structure and bond relationships that may help in understanding its biochemical properties. It also gives us hope for the discovery of newer agents that are having historical significance in treating human health and diseases and curbing the menace of antibacterial resistance and the discovery of antimicrobials.

## ACKNOWLEDGEMENTS

Authors acknowledge,

Dr. K. Vijay Kumar, Dean (Science and Technology), Chairman (Department of Zoology) Gulbarga University Kalaburagi, for his support in carrying out this research

Dr. Y. Vishwanath, Medical Superintendent (TB sanitorium), Professor, Department of Pharmacology and Faculty of Department of Pharmacology, Vijayanagar Institute of Medical Sciences, Ballari for their support and interest in the project.

Dr. Sampath, Prof. and Head, Department of Microbiology, Dr. Kirtilaxmi K. B. Assistant Professor, Department of microbiology, Raja Rajeswari Medical College and Hospital, Bengaluru, Karnataka, India. For their support with regards to providing of laboratory setup, obtaining ATCC strains of bacteria and fungi and working as a team to make this study successful.

## Conflict of Interest

The authors do not have any competing conflict of interest.

## Ethical Standards

This procedure did not involve any endangered or protected species, and the method of collection of samples was in accordance to the ethical standards and guidelines of the in-house research committee.

## Sponsorship and Financial Aid

The authors have not received any grants, sponsorship or financial aid in any form for carrying out the proposed research; all the costs are borne by the researchers themselves.

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